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PRODUCTION AND VALIDATION OF AN IgM ANTIBODY FOR THE DETECTION OF SPORE-FORMING BIOAGENTS

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14. ABSTRACT
Antibodies are the essential components in biosensors that specifically bind biological agents, providing a capability essential to fulfilling the defense mission of Department of Defense. An IgM monoclonal antibody specific for the spore forming *Bacillus anthracis* has been developed by the John Kearney Laboratory (University of Alabama, Birmingham, AL). However, a means of purifying IgM in quantities sufficient for testing and evaluation was not readily available. The IgM has a monomeric subunit structure consisting of two heavy and two light chains. However, IgM is efficiently secreted only if it is polymerized into either pentamers or hexamers, 750 - 900K Daltons. Unlike IgG, IgM is not effectively purified by either protein-A or protein-G. These properties introduce difficulties in the IgM antibody purification. In this study, a platform process was developed for the purification of IgM, including an immuno-affinity chromatography method using a goat anti-mouse IgM antibody coupled to Sepharose as a polishing chromatographic step. The purified IgM was active as determined by ELISA.

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PREFACE

The work described in this report was authorized under the Technology Transition Program, Joint Science and Technology Panel for Chemical and Biological Defense. This work was started in May 2002 and completed in May 2003.

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PRODUCTION AND VALIDATION OF AN IgM ANTIBODY FOR THE DETECTION OF SPORE-FORMING BIOAGENTS

1. INTRODUCTION

A biological agent of great concern is *Bacillus anthracis*, the causative agent of anthrax. It is well known that immunological methods for detecting *B. anthracis* spores and distinguishing these from spores of closely related *Bacillus* species are lacking. Monoclonal antibodies (mAbs) are the essential components in biosensors that specifically bind to biological threat agents, providing a capability essential to fulfilling the biological defense mission of Department of Defense.[1, 2] An adequate supply of mAb against threat agents allows the Department of Defense to fulfill its biological defense mission. An immunoglobulin M (IgM) monoclonal antibody (mAb) specific for *B. anthracis* has been developed by the laboratory of Dr. John Kearney (University of Alabama, Birmingham, AL).[3, 4] However, a means of purifying IgM in quantities sufficient for testing and evaluation was not readily available. The IgM antibody is secreted as multi-subunit polymers that consist of as many as three discrete polypeptides: m heavy chains, light (L) chains, and joining (J) chains. Like other immunoglobulins, monomeric IgM has a basic subunit structure consisting of two heavy (H) and two light (L) chains. However, IgM is efficiently secreted only if it is polymerized into pentamers or hexamers, which comprise five or six covalently associated μ2L2 monomeric subunits, respectively, ~750K-900K Daltons (see Figure 1).[5] Furthermore, unlike IgG, IgM is not effectively bound by protein-A or protein-G (J.T. Park and F.J. Kragl, unpublished data 2003). These properties introduce difficulties in the purification and analysis of IgM.

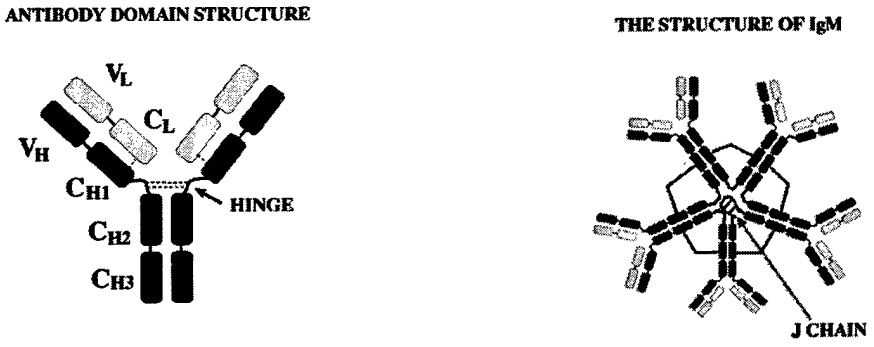

Figure 1. Illustration of Antibody (Monomeric) Structure ~150kD and IgM (Pentameric) Structure ~750-900kD

In this study, a platform process was developed for the cell culture and purification of IgM including an immuno-affinity chromatography method using a goat anti-mouse IgM antibody coupled to Sepharose as a polishing chromatographic step. Using this platform process we produced a purified IgM antibody. Additionally, the

biological activity of purified IgM antibody was tested by an enzyme-linked immunosorbent assay (ELISA).[2]

2. MATERIALS AND METHODS

2.1 Materials.

Becton Dickinson (BD) Cell mAb Medium Quantum Yield (BD Cell Media) was purchased from BD Bioscience (Sparks, MD) and fetal bovine serum (FBS) was purchased from Invitrogen Corp. (Carlsbad, CA). The T-25, T-75, and T-150 flasks were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). Two-liter gas permeable bags were purchased from TC Technologies (Minneapolis, MN). Opticap disposable filters were purchased from Millipore Corporation (Bedford, MA). Hollow fiber membrane cartridge (100K Daltons ultrafiltration) and Sephadex G-25 desalting resins were purchased from Amersham Biosciences (Piscataway, NJ). The preparative-scale and analytical gel permeation chromatography (GPC) columns were purchased from Tosoh Bioscience (Montgomeryville, PA). The XK26 and XK50 empty columns were purchased from Amersham Biosciences. Goat anti-mouse IgM antibody linked to Sepharose was obtained from Southern Biotechnology Association (Birmingham, AL). Trizma base (T-6066), Trizma hydrochloride (T-5941), sodium chloride (S-9888), and sodium azide (S-8032) were purchased from Sigma Chemical Company (St. Louis, MO). Sodium citrate dihydrate (catalog #3649) and citric acid monohydrate (catalog #0119) were purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals used are at least American Chemical Society (ACS) grade.

2.2 IgM Antibody Production Process.

Figure 2 illustrates the schematic process diagram of the IgM production. The IgM was produced using hybridoma cells, which were grown up in BD Cell Quantum Yield media with 10% FBS, 2 mM glutamine, and 1 mM Pyruvate.[2, 6] The cell culture was grown using 2-L gas permeable bags (see Figure 3).[6, 7] The cell culture, at harvest, was clarified using a high capacity 0.22 μm (Opticap 4") inline filter from Millipore. After clarification, the cell culture supernatant was concentrated using 100 kD hollow fiber tangential flow filtration (TFF). The concentration retentate containing the IgM was then injected onto preparative-scale GPC (3000SW/4000SW) columns. Once the fractions from the GPC were pooled together, a small portion of the pool was injected onto an anti IgM coupled sepharose column. Antibody-containing fractions were pooled, and a buffer exchange into the final product buffer, phosphate buffered saline (PBS) at pH 7.2 including 0.05% sodium azide, was performed by a Sephadex G-25 desalting column. Finally, antibody solutions were sterile-filtered by a 0.22 μm disposable filter.[2, 8, 9].

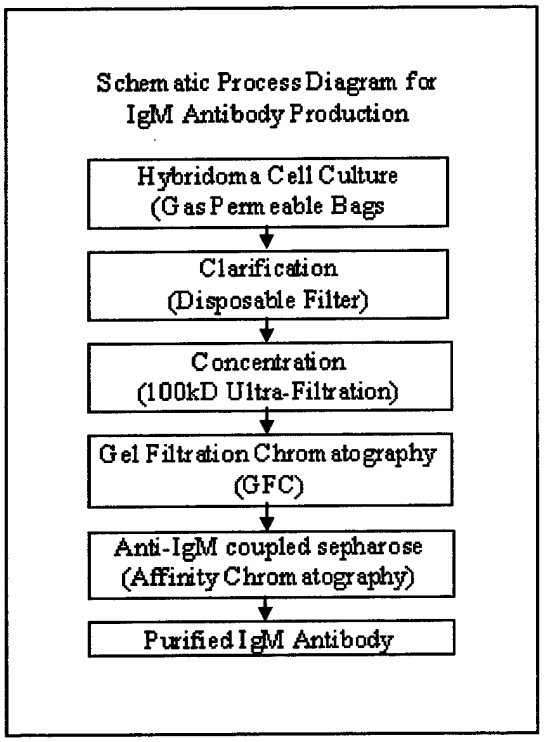

Figure 2. Schematic Process Diagram for IgM Antibody Purification

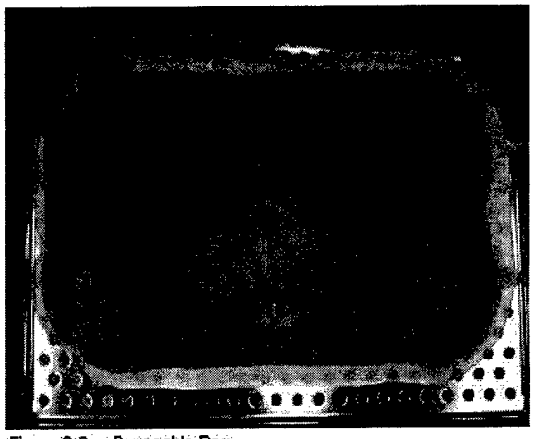
Figure 3 Gas Permeable Bag

Figure 3. Picture of 2-L Gas Permeable Cell Culture Bag

2.3 Analytical Methods.

Goat anti-mouse IgM antibody linked to Sepharose was obtained from Southern Biotechnology Association (Birmingham, AL) and empty HPLC columns (4.6 mm ID x 50 mm L) were obtained from Applied Biosystem (Foster City, CA). Analytical gel permeation chromatography (GPC) TSK_{SWXL} guard column (part #08543) and TSK $G3000_{SWXL}$ (part #08541) were purchased from Tosoh Bioscience (Montgomeryville, PA). Mouse IgG and Bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, MO). Sodium phosphate monobasic dihydrate and sodium phosphate dibasic heptahydrate were purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals used are at least ACS grade.

Trypan blue method was used for the cell count and viability measurement as described elsewhere.[2] Fifty microliters of cell suspension was removed from the total volume of cell chamber contents and diluted with 450 µL of complete medium. Fifty microliters of this suspension was diluted with 50 µL trypan blue dyes. This was applied to a hemacytometer and four fields of 16 squares were counted. Dead cells were blue and viable cells were white/clear.

The HPLC system used for the assay consists of a HPLC pump (Beckman Coulter, System Gold Model 118) (Beckman Coulter, Fullerton, CA), an autosampler

(Beckman System Gold Model 508), and an UV detector (Beckman Coulter, System Gold Model 166). Chromatographic data were acquired and analyzed using 32-Karat software (Beckman Coulter). An anti-IgM mAb column was packed using an empty HPLC column (0.8 mL) as described elsewhere.[9, 10] For each analysis, samples were pretreated by filtering with 0.2-μm filters or micro centrifuging before injection. After injection, the phosphate buffered saline (PBS) buffer at pH 7.2 (20 mM sodium phosphate/150 mM sodium chloride) was used as a loading/washing buffer at the rate of 0.5 mL/min for 10 min. A target mAb was eluted with the 100 mM glycine buffer at pH 2.0 using a linear gradient at a rate of 0.5 mL/min for 10 min. A HPLC system similar to that mentioned above was used for the measurement of mAb purity. Samples were injected through a single GPC TSK $G3000_{SWXL}$ column. The purity of mAb was measured by 32-Karat software.

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a 4-15% gradient gel was used. The gel and gel running apparatus were purchased from BioRad (Hercules, CA).

3. RESULTS

3.1 Production of Purified IgM Antibody.

The IgM was produced *in vitro* using hybridoma cells, which were grown up in BD Cell Quantum Yield media with 10% FBS, 2 mM glutamine, and 1 mM Pyruvate. The cell culture was grown in 2-L gas permeable bags. The gas permeable bags were placed in an incubator at 37 °C, using CO_2 for pH control. The bags were incubated in a 37 °C CO_2 incubator for approximately 1 month. During the cell culture incubation, the cells grew in number from 1.08×10^5 cells/mL at inoculation to 4.95×10^6 cells/mL at time of harvest. During the incubation period, the percent viability of the cells declined from 92.6% at inoculation to 9.1% viability at time of harvest. As the viability of the cells decreased, the concentration of IgM product in the cell culture media increased to a final concentration, 0.514 mg/mL-media at time of harvest (see Figure 4).

The cell culture media was clarified using a 0.22 μm disposable filter. This filter provided a large surface area, so that it would not readily be fouled by the cells, which were suspended in the cell culture media. This filtration step removed the cells and cell debris from the cell culture media, allowing the media to be further processed. Once the media was clarified, the pH was adjusted to 7.2. A final concentration of 0.05% sodium azide was also added to the media to inhibit bacterial growth.

After clarification, the cell culture supernatant was concentrated using a 100 kD hollow fiber TFF membrane. The cell culture media was concentrated from an original volume of 11,000 mL to a final volume of 710 mL. Another advantage of the concentration step was that the membrane allowed cell culture contaminants such as BSA to permeate through the membrane while retaining the IgM, a substantially larger protein, in the filter retentate.

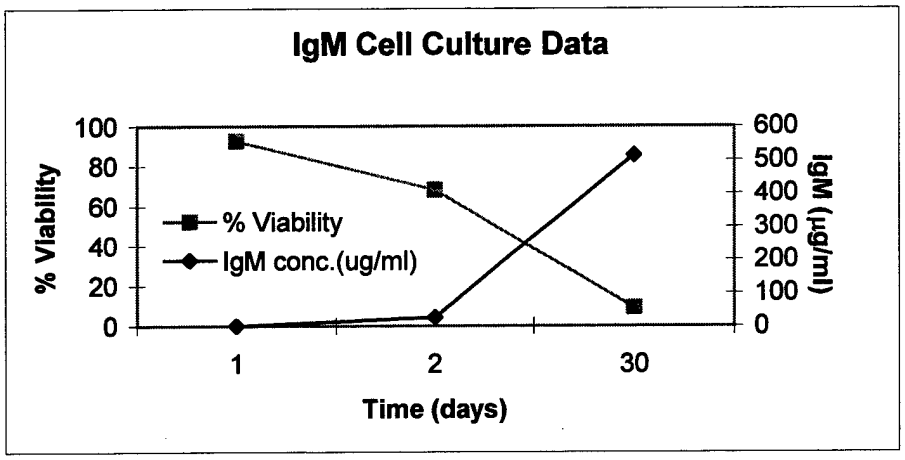

Figure 4. Cell Culture Data. Graph Representing the Percent Viability of the Cells Compared to the IgM Concentration in the Cell Culture Media.

The retentate from the concentration step was injected onto prep-scale GPC columns. The maximum injection volume of the GPC was 20 mL. This required the retentate pool to be injected onto the GPC a total of 35 times as shown in Figure 5. Fractions were taken from each of the 35 GPC purifications, and were later pooled together and analyzed using analytical GPC, and analytical anti-IgM affinity column.

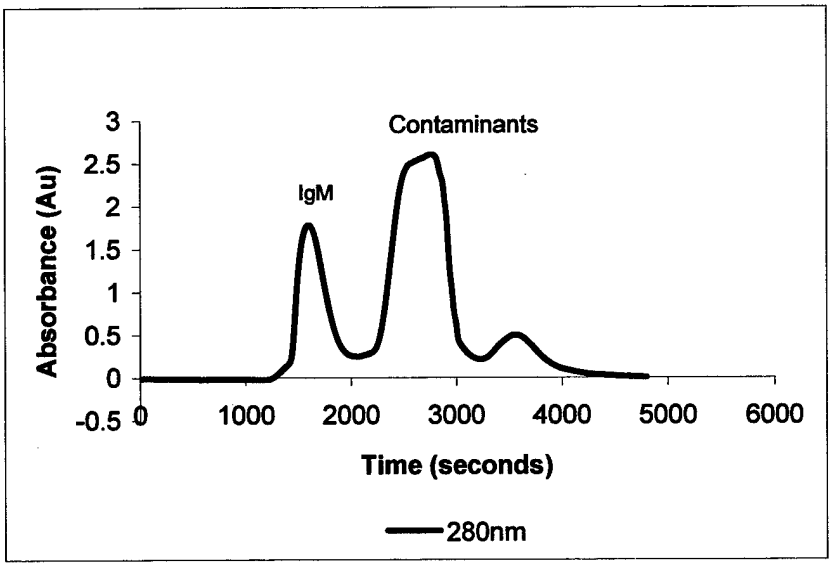

Figure 5. Chromatogram of Prep Scale GPC Purification of IgM Antibody.

From this pool, 1 mL of the GPC pool was injected onto an anti-IgM coupled sepharose column as shown in Figure 6. Fractions from the Anti-IgM column purification were taken and analyzed.

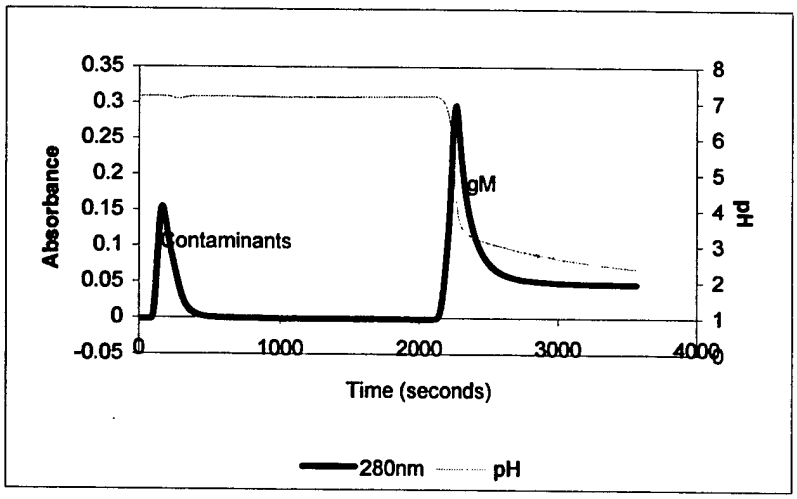

Figure 6. Chromatogram of IgM Antibody Purification Using an Anti-IgM Antibody Coupled Sepharose Affinity Chromatography Step.

3.2 Analysis of IgM Antibody Samples.

Each of the in-process samples was studied, using three analytical methods. The first method used for analysis was an immuno-affinity chromatography method which used a goat anti-mouse IgG (IgM specific) coupled sepharose resin. The IgM product was bound to the resin at a neutral pH (pH 7.2) and allowed other contaminants to pass through the column. The column was then washed using the neutral pH buffer (Phosphate buffer pH 7.2) allowing all of the unbound contaminants to pass through the column. The IgM was then eluted from the column at a low pH (Glycine buffer pH 2.0). This allowed quantitative calculations to be performed on the IgM product purity. The second method used for analysis was an analytical GPC. The IgM product was injected onto the GPC column at a neutral pH (Phosphate buffer pH 6.5). This column separated the IgM from other contaminants based on size (i.e., molecular weight). This method was used to analyze the IgM product purity. The third method used for analysis of the IgM was SDS-PAGE. The IgM from various steps in the purification process was run on a 4-15% gradient acrylamide gel. An IgM standard was also run on the gel to confirm the presence of IgM in the process samples.

An immuno-affinity HPLC column was employed for the quantitative analysis of the IgM in-process samples obtained during production process as shown in Figure 7. The IgM crude culture had a purity of 6.0%. The IgM purity after the GPC purification step was 72.3%. The purity of the IgM after the Prep anti-IgM Antibody coupled sepharose column step was 93.1%. The IgM product purified by the purification process through the prep-scale GPC had a yield of 22%, as determined by analysis with the immuno-affinity HPLC.

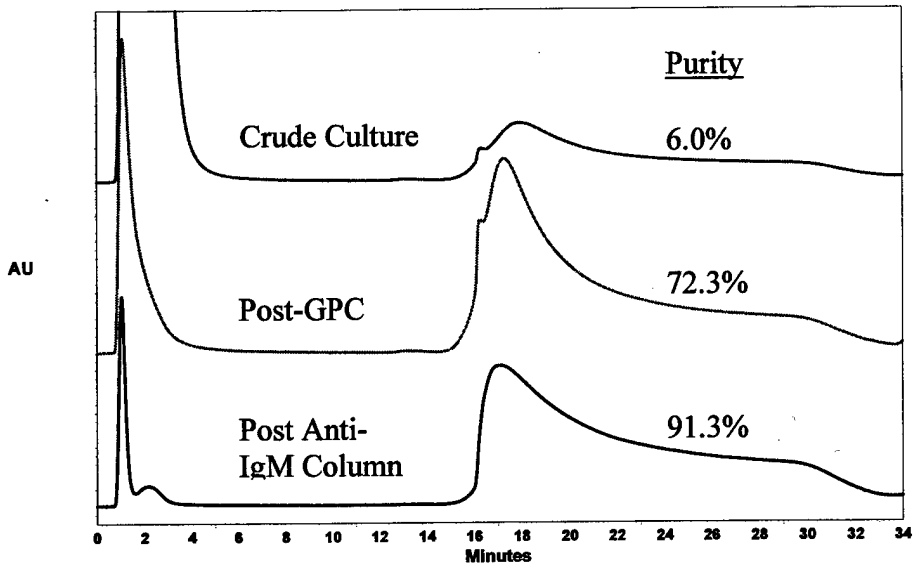

Figure 7. Chromatograms of IgM Purity Shown from Process Samples Analyzed by an Immuno-Affinity HPLC Method.

The IgM in-process samples were also analyzed using SDS-PAGE using a 4-15% gradient gel as shown in Figure 8. The purity of the IgM in each purification step can be seen on the gel. Five samples were run on the gel. First sample was the molecular weight standard. The second sample was the IgM standard purchased from Sigma. The third sample was IgM in crude cell culture before purification. The fourth sample was IgM after injection onto the prep scale GPC. The fifth sample was IgM after injection onto the anti-IgM antibody coupled sepharose immuno-affinity column. The SDS PAGE gel is a good analytical method to show the increase in IgM purity throughout purification process.

Figure 8. SDS-PAGE Results of the IgM In-process Samples. Lane #1: Molecular Weight Ladder, Lane #2: Sigma IgM Standard, Lane #3: Crude Cell Culture, Lane #4: Post GFC Purified IgM, Lane #5: Post Anti-IgM Column Purified IgM.

Analytical GPC column was used to quantitatively analyze the IgM purity of the product pool from the prep-scale GPC chromatography fractions. The chromatogram from the analytical GPC injection showed that the product pool from the GPC purification had a purity of 83.7% and that the retention time of the IgM differed sufficiently from other contaminants such as BSA or IgG (Figure 9).

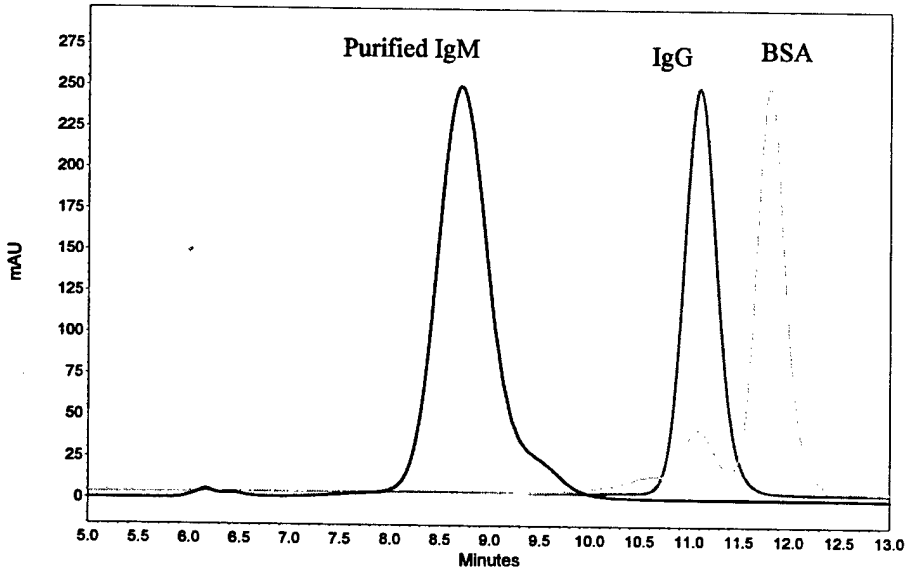

Figure 9. Chromatograms of Final IgM Product, IgG Standard, and BSA Standard Obtained from Analytical GPC Column.

An ELISA was used to determine the activity of the IgM antibody. The antibody was shown to be active against the *Bacillus anthracis* antigen as shown in Figure 10. The ELISA showed that this IgM antibody may be a good candidate for use in biosensors for the detection of *B. anthracis* spores.

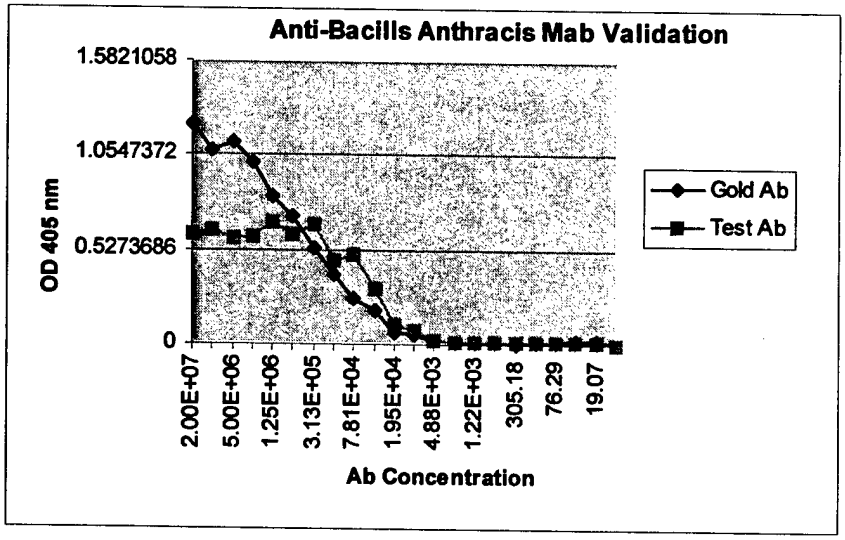

Figure 10. ELISA Data of the Purified IgM Product Against an IgG Antibody Standard.

4. CONCLUSIONS

The IgM antibody DH4-1 was successfully produced and purified using the platform process, which was developed here. The IgM was produced with sufficient purity and concentration that it may be used in biosensors for the specific detection of *Bacillus anthracis* spores. The purity of the DH4-1 IgM product was confirmed using three different analytical techniques, (immuno-affinity chromatography, GPC, and SDS-PAGE). The activity of the IgM antibody was measured and confirmed using ELISA. The analytical anti-IgM antibody coupled sepharose resin immuno-chromatography method was an invaluable tool for the analysis of the IgM antibody. With this technique, the IgM purity could be quantitatively determined in the purified sample and even in complex biological media, such as cell culture and in-process samples.

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